THE DIGESTION AND ABSORPTION OF FAT IN LAMELLIBRANCHS [1]

W. C. GEORGE

Department of Anatomy, University of North Carolina, Chapel Hill, North Carolina

The problem of digestion in lamellibranch mollusks has been a subject of interest to biologists for many years. It seems well established that the digestion of carbohydrates takes place largely extra-cellularly in the lumen of the stomach through the action of enzymes liberated through the dissolution of the crystalline style. The place and mode of digestion and absorption of proteins and fats have occasioned disagreement. One concept supported especially by a series of papers by Yonge (1923, 1926a, 1926b, 1930, 1946) holds that the digestion of fats and proteins is exclusively or largely intra-cellular, occurring in phagocytes of the blood and in cells of the digestive diverticula. Another group of investigators has questioned the correctness of this view and has presented some evidence to indicate that the digestion of fats and proteins occurs extra-cellularly through the action of enzymes of uncertain source (Swano, 1929; Nelson, 1933; Mansour, 1945, 1946; Mansour-Bek, 1945, 1946).

The present author's concern with the structure and primitive functions of blood and hemolymph gives the problem of digestion in invertebrates a special interest. The thesis of intra-cellular digestion in leucocytes was appealing and tentatively accepted (George, 1941). That digestion by phagocytes of the blood was so extensive as to be a significant feature of the digestive process did not appear soundly enough established, however, to justify its acceptance as a basis for generalization. The present investigation, which is limited to the digestion and absorption of fats, was undertaken during July–August, 1950, to determine the extent of participation of leucocytes in the digestive process of some lamellibranchs.

Material and Methods

Several species of lamellibranchs were used but principally the common oyster, *Crassostrea virginica,* and the ribbed mussel, *Modiolus demissus,* both abundantly available in Beaufort Harbor. Stained neutral fats (olive oil and peanut oil) were fed as emulsions stained with Sudan black B or with Sudan III (Scarlet red). Scarlet red,[2] which stains both neutral fats and fatty acids pink, was chosen for most experiments because of its advantage in subsequent counter-staining with Nile blue sulfate. Emulsions of the oil, previously stained to saturation, were prepared as follows: 60 cc.

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[2] The scarlet red used in this investigation was given to the author a few years ago by the late Simon Henry Gage and is from the lot used by him and Fish in their investigations.

of the stained oil, 15 grams of powdered acacia and 30 cc. of distilled water were put in a Waring Blendor and thoroughly mixed. The emulsion was then thinned somewhat by the gradual mixing in the Blendor of 20 cc. additional distilled water. This produced a satisfactory stock emulsion with particles ranging in size from less than 1 μ to perhaps 10 μ. Oil immersion examination of a diluted drop of the stock emulsion shows the larger droplets to have a clear pink color; a slight but definite pink is recognizable in medium sized droplets; no definite color is detectable in individual droplets of the smallest size. In some cases the emulsion was inserted with a pipette through the mouth into the stomachs of animals after the removal of one valve. Sometimes animals were fed in salt water aquaria. A few drops of the emulsion poured into the water, kept turbulent by bubbling air through it from an air compressor, is quickly distributed throughout an aquarium. As some of the oil slowly accumulates at the surface, this can be skimmed off and the concentration restored by the addition of fresh emulsion.

Fat droplets withdrawn with a pipette from the stomachs at definite times after feeding, or droplets taken from other experimental material can be identified by color when examined with the microscope. By counter-staining with a solution of Nile blue sulfate allowed to run under from the edge of the cover glass, one can determine if any hydrolysis of neutral fat has occurred. Droplets that have undergone reduction to fatty acid are changed from pink to a clear blue as the Nile blue sulfate reaches them, whereas droplets of neutral fat become deeper pink (an orange pink). The same microscopic picture is obtained after exposure of emulsion to a solution of commercial lipase (steapsin).[3] No droplets of the untreated stock solution turn blue when counter-stained with Nile blue sulfate; on the contrary they all turn a deeper pink. As a counter-stain, concentrations of Nile blue sulfate ranging from 1 : 1000 to 1 : 300,000 were used and proved effective. A solution of 1 : 10,000 was settled on as most satisfactory.

Tests for lipase in styles and various other structures were made by mixing small quantities of stock emulsion with a mince of the structures in 2–3 drops of sea water or tap water on a hollow ground slide and subsequently counter-staining a sample with Nile blue sulfate. Generally no bactericidal agent was used; similar results were obtained when a drop of 0.5% phenol solution was added to 2 drops of water of the mince.

Material for sections was fixed in 10% formalin in sea water.

Results

Oysters and mussels kept for as long as five days in turbulent water with a suspension of olive oil emulsion stained with Sudan black showed no deposition of stained fat in any of the storage tissues although the emulsion was freely taken into the gut. Similar experiments with emulsions of peanut oil stained with scarlet red likewise gave no evidence for storage of stained fat such as Gage and Fish (1924) found in mammals and birds.

Although these experiments gave no evidence for storage of experimental fat in the tissues, the evidence for digestion within the stomach seems clear. In emulsion withdrawn and tested after having remained in the stomach for only a short time, some droplets became a pale blue after Nile blue counter-staining, indicating the

[3] Lipase (steapsin) from Nutritional Biochemicals Corporation was used.

beginning of fat digestion, but many droplets retained the pink color of neutral fat. In emulsion that had remained in the stomach for several hours, the number of blue droplets after Nile blue sulfate was much greater and the depth of blue was deeper, most drops showing an intense blue color. In emulsion withdrawn and tested after a still longer time in the stomach, some of the larger droplets had a shrunken appearance, due, perhaps, to the diffusion of glycerol from the drops; and after 30 hours exposure to stomach juice some droplets contained needle-like crystals, probably fatty acid crystals.

My findings with regard to the digestion of fat in the stomach seem to be in conflict in some essential matters with the conclusions of Yonge (1926b), who fed olive oil stained red with Nile blue sulphate to oysters. On p. 351 he says, "In the lumen of the stomach there were immense numbers of phagocytes, most of them with ingested oil. In certain cases they collected in great numbers round large droplets of oil, which had turned blue under the influence of their enzymes. *All oil droplets lying free in the stomach and not surrounded by phagocytes retained the red color*—evidence of the absence of lipase in the stomach." The presence of phagocytes and their ingestion and digestion of oil I can confirm; but the absence of digestion of oil other than under the influence of phagocytes is not in accordance with my observations. It is conceivable, of course, but not likely that *Ostrea edulis* and *Crassostrea virginica* differ markedly in their physiology of fat digestion and absorption, but it seems more probable that the oil observed by Yonge had been inadequately exposed to the action of stomach juices.

If the disintegration of leucocytes liberates appreciable amounts of lipase or if they secrete it, it seems reasonable to assume that some should be present in the plasma. To test for lipase in plasma, fat emulsion diluted with sea water was injected into the hearts of several oysters and mussels and one clam. In all cases, tests for fat digestion after two to ten hours were positive so far as digestion within blood cells was concerned and negative for extra-cellular digestion in plasma. Also, hanging drop cultures of fat emulsion mixed with blood were made. Examination after one hour showed ingestion and digestion of pink fat droplets within leucocytes. No evidence for fat digestion could be found in the droplets that remained in the plasma. Further tests for lipase in plasma were made during August, 1951, by placing drops of plasma from fresh water mussels, salt water mussels and oysters upon smears of margarine, unstained peanut oil, and Sudan III-stained cotton seed oil. This was to check the technique and results of Yount (1950), who reported the digestion of neutral fat by the plasma of oysters, mussels and clams. My several experiments were negative, no droplet ever turning blue after Nile blue except in the case of one batch of oil stained with Scarlet red the previous summer and carried over. In smears from that lot of oil, some blue droplets appeared after counter-staining with Nile blue. It turned out, however, that blue droplets appeared outside the areas to which plasma was applied as well as inside them and, indeed, in smears to which nothing was applied except Nile blue. Evidently that particular batch of oil was rancid, and it seems possible that Yount's positive results were due to rancid fat in his margarine. These observations seem to establish two points: 1) the leucocytes produce their own lipase, and 2) there is no evidence that they liberate any into the plasma (or, presumably, into stomach juice).

To determine the source of the lipase of the stomach juice, a drop of fat emulsion was added to minced crystalline style in two or three drops of sea water. Without

exception these tests were positive for lipase activity. The specimens tested consisted of 12 oysters, 15 mussels, one clam (*Venus mercenaria*), one *Atrina livida,* and one razor clam (*Ensis directus*).

Two typical case reports from my notes will illustrate the progress of digestion. *Mussel MA*—style was removed, washed, and minced in 2 drops of sea water; a drop of emulsion was added at 9:15 A.M. At 11 A.M. tests with Nile blue sulfate showed 10–15% of oil droplets to be blue; at 12 M. about 25%; at 2:30 P.M. almost 100%. *Oyster 27-B*—style minced in sea water at 11:45 A.M. At 12:30 P.M. 75% of the droplets are blue after Nile blue; at 4:45 P.M. 100% are blue. There is variation among individual oysters and mussels in the speed of the digestive action, or in the percentile response of fat droplets. These experiments indicate that in all these species of lamellibranchs the style is an important source of lipase.

To determine if active lipase was present in the digestive diverticula, fat emulsion was mixed with mince of a chunk of the glandular mass in two or three drops of sea water. Tests with Nile blue sulfate were made after varying times with the following results: all 9 oysters tested and the single *Atrina rigida* were negative for fat digestion; all 10 mussels tested and the single clam and the razor clam were positive. In the case of one mussel, the lining membrane of the stomach and the glandular mass were dissected apart, minced and tested separately. The glandular mass was positive and the lining membrane was negative for lipase. Although the tests for fat digestion by mince of the digestive diverticula were clearly positive in some species, they were less strongly so than was the case with the styles. The conclusion seems justified, then, that the principal source of lipase for extra-cellular digestion in the stomachs of the lamellibranchs studied is in the crystalline styles. Apparently this source may be supplemented in some species with lipase secreted by glandular tissue of the digestive diverticula.

Observations were made to determine if fat is transported from the lumen of the gut into the tissues by free phagocytes as supported by Yonge (1926b) and Takatsuki (1934). It seems reasonable to expect to find some if they are to be looked upon as significant agents in digestion and absorption. Oysters that had been kept in a suspension of stained fat emulsion in sea water were sacrificed at 8, 18, 21, 22, 25, 29, and 30 hours. Considerable fat was in the guts of these animals. Careful examination revealed no leucocytes with ingested experimental fat when blood was withdrawn from the hearts. In most specimens a few cells were found with apparent oil droplets, but no pink color was identifiable. These droplets stain blue with Nile blue, as do fatty acids (and intrinsic granules of oyster blood cells). In the connective tissue surrounding the gut epithelium, free cells with globules of a probably fatty nature were present but in no case could it be determined that they contained experimental fat. On the basis of the evidence one could say only that the blood cells may possibly transport a small amount of fat into the blood and tissues but the amount of transport is not enough to be significant in total nutrition.

Is the neutral fat used in these experiments absorbed at all and if so, how? To get an answer to these questions, sections of stomachs of experimental and control oysters were made by the freezing technique. Examinations of unstained sections from animals fed fat emulsion showed abundant small granules or droplets in the ciliated epithelium of the stomach and in the ciliated epithelium of the ducts of the digestive diverticula. Under high powers of the microscope these droplets individually show no color; but under low power they give a diffuse pinkness to the epi-

thelial layer. There is little doubt that they are from the stained fat fed the animals. A secondary staining of the section with an alcoholic solution of Sudan III colors these droplets a bright orange. Counter-staining with Nile blue sulphate colors them a deep blue. The conclusion seems justified that they are droplets of the stock emulsion reduced to fatty acid and in process of being absorbed through the epithelium. After secondary staining with Nile blue or Sudan III it is clearly seen that the droplets are most abundant in the apical one-third to one-half of the columnar epithelial cells and least abundant in the middle third, a distribution accounted for in part, at least, by the fact that the nuclei occupy a large part of the middle third of the epithelial layer. Cells of some areas of the mucosa contain more droplets than other areas.

Frozen sections of control animals, neither fed nor starved, showed some sudanophil droplets in the epithelium but no such great accumulation as in the case of the animals fed oil.

What appear to be blood cells are to be found sometimes in the epithelium of both experimental and control animals. They are not present in sufficient numbers, however, as to justify corroboration of the conclusions of Yonge (1926b) and Takatsuki (1934) that wandering phagocytes are important agents of absorption in the gut. On the contrary, the facts from these experiments seem to lead to the conclusion that neutral fat fed to these lamellibranchs is reduced to fatty acid in the lumen of the gut and absorbed as independent particles through the ciliated epithelium of the gut and ducts of the digestive diverticula, and that little if any is carried through by leucocytes.

The epithelium of the tubular alveoli of the digestive diverticula shows two types of cells which tend to be associated in groups: 1) cells with coarse to fine granules of greenish brown color, and 2) smaller cells with smaller colorless granules of approximately uniform size, which Yonge (1926a) considers to be immature cells. Neither of the types of granules in these cells stains with alcoholic Sudan III. After Nile blue sulfate the greenish-brown granules become deep green; the colorless granules found in the smaller cells do not stain. No fat droplets could be discovered within or between the epithelial cells of the tubular alveoli as were found in the ciliated epithelium of their ducts and of the stomach. Hence, the results of feeding emulsified oil to oysters provide no evidence for the ingestion or absorption of fat particles by the epithelium of the digestive tubules.

What evidence do these experiments reveal concerning the storage of fat in the connective tissue around the gut? The connective tissue of lamellibranch mollusks contains large polygonal vacuolated cells with centrally located nuclei having a zone of perinuclear cytoplasm with strands radiating outward to a zone of peripheral cytoplasm. The vacuolar spaces within these cells contain few or many droplets of various sizes which in unstained sections are colorless. Sudan III in 70% alcohol colors them bright orange; Nile blue sulfate colors them blue. They are dissolved by higher alcohols and xylene. They probably contain stored fatty acid, but in unstained frozen sections of experimental animals no pink color could be detected in these droplets to identify them as having been derived from the stained emulsion fed the oysters. In addition to the fixed vacuolated cells of the connective tissue, large wandering cells are present. They contain drop-like inclusions of different sizes ranging in color from a clear brown to a pale color that might be mistaken for pink. They contain in addition small, relatively uniform

granules resembling the intrinsic granules of cells of the blood. Droplets and granules both stain a blue green with Nile blue sulfate but they do not stain with alcoholic Sudan III. They are not dissolved by alcohols and xylene, and they are present in animals not fed fat emulsions as well as in those that are. It is suggested that these inclusions are not neutral fat or fatty acid but probably chromolipoids, perhaps mixed with other substances.

Discussion

Evidence from this investigation for the extra-cellular digestion of fats in the stomachs of oysters and mussels seems unequivocal and conclusive. Previous evidence has been contradictory. Claude Bernard (1855) reported that the stomach juice of oysters digests starches and fats. Albrecht (1921) found that extracts of the intestinal tracts of the clam, *Tivella stultorum,* have lipolytic action. More recently Mansour-Bek (1945) reported lipolytic activity by digestive juice pipetted from the stomachs of *Tridacna elongata* and *Pinctata vulgaris.* On the other hand, Yonge (1926a, 1926b, 1930, 1937, 1946) supports the thesis that in lamellibranchs digestion of proteins and fats is exclusively intra-cellular, the only extra-cellular enzymes normally present being those set free when the head of the crystalline style is dissolved in the stomach. He finds these enzymes to be exclusively amylolytic (Yonge, 1923, 1926b). Any proteolytic or lipolytic action found in filtered stomach juice is attributed to cytolysis of phagocytes. In this view he is supported by Graham (1931). (Other pertinent references are to be found in the papers cited above.)

Correlated with the differences of opinion in regard to intra-cellular and extra-cellular digestion in lamellibranchs is a corresponding lack of harmony concerning the enzymatic activity of the crystalline style and of the digestive diverticulum. It has become well established that the dissolution of the style liberates amylolytic enzymes. With regard to other enzymes the observations of workers are at variance. Yonge in a number of papers contends that the style consists largely of amylolytic enzymes and that no other enzymes are present in it. Chestnut (1949) with the methods he used found amylolytic but no demonstrable proteolytic or lypolytic activity in extracts of the crystalline style. Some other investigators get results not entirely in accord with Yonge's. Sawano (1929) reported the presence of lipase as well as amylase and protease in extracts of the crystalline style. Fox and Marks (1936) also attributed lipase as well as carbohydrases to extracts of the style of *Mytilus.* In discussing the digestion of animal forms by the oyster, Nelson (1933), without specifying the nature or the source of the active substances, states that "It is evident therefore that some substance or substances present in the stomach of an oyster with a well formed style can penetrate the chitin of crustacea and the cuticle of nematodes resulting in death and disintegration of the animals." Mansour (1946) and Mansour-Bek (1946) seem to consider that globules pinched off or shed from cells of the digestive gland are the source of extra-cellular lipolytic and proteolytic enzymes.

The experiments and observations reported in a previous part of this paper have an important bearing on certain features of these arguments. Sensitive tests show that hydrolysis of neutral fats does occur extra-cellularly in the cavity of stomachs of oysters and mussels. These tests also give a positive reaction for the presence of

lipase in aqueous extracts of the crystalline style, which appears to be the principal source of the enzymes that mediate the hydrolysis of fat in the stomach. It is possible that lipolytic enzymes from the digestive diverticulum supplement those from the style, since minced diverticulum in water gives a positive reaction for the presence of lipase in individuals of some species, not all. One should not overlook the possibility, however, that normally this demonstrated lipase from the digestive gland might never become an extra-cellular enzyme but function intra-cellularly.

It is still an open question whether the tubules of the digestive gland are secretory or whether they exclusively absorb and digest phagocytically. A detailed exploration of the functions of the digestive gland was not a part of this investigation. However, some observations are significant. We have seen that the gland contains, possibly secretes, lipase in some species, but none was demonstrated in the oysters of these experiments. Chestnut (1949), working with the same species of oyster, found that extracts of the digestive diverticula have lipolytic activity as well as amylolytic and proteolytic activity. This difference in results suggests the possibility of seasonal variation in the presence of active enzymes or perhaps a difference in the effectiveness of different techniques in extracting enzymes from the cells. Phagocytic action is suggested by the fact that cells of the tubules contain what appear to be bits of foreign debris and greenish-brown granules of an organic nature that may have been ingested but probably were manufactured. These experiments produced no positive evidence and little circumstantial evidence that cells of the tubules ingest fat emulsion. It could be that the tubules of the digestive diverticula are specialized for the phagocytosis or absorption of some substances, not of others. Many authors have attributed an absorptive function to them. Saint-Hilaire (1893) found that the cells of the tubules of some lamellibranchs and other mollusks are not ferment cells but absorptive cells. Enriques (1902) and Gutheil (1912) consider that they absorb but that their main function is excretory. List (1902) found that in *Mytilus* particles of India ink were taken from water containing it by cells of the digestive diverticula. Vonk (1924) got similar results with oysters. From microscopic examinations of glands during the later stages of digestion in *Ostrea* and *Modiolus,* Nelson (1925) was led to believe that in these forms even more food is digested in the digestive glands than in the stomach. Yonge (1926a), by feeding iron saccharate to various lamellibranchs, found that the substance was taken up by amoebocytes and by cells of the tubules of the digestive diverticula but by no other part of the gut. Coe and Fox (1944) state that in *Mytilus californianus* "Many of the smallest objects and particles are phagocytized by the cells lining the digestive diverticula."

As regards the processes of absorption of fat, the observations here reported indicate that it is largely absorbed as particulate matter through the ciliated epithelium of the gut and the ducts of the digestive diverticula. None was identified in the cells of the tubules themselves. All fat observed in the process of being absorbed gives the reaction for fatty acid. There is no evidence for or against absorption as soluble fat, although it seems reasonable to assume that the glycerol component is so absorbed. Although leucocytes free in the lumen of the gut may ingest fat droplets in quantity, the evidence does not indicate any considerable transport of fat by them into the tissue since no leucocytes with identifiable experimental fat could be found in the blood or tissues. Furthermore, in confirmation of the ob-

servations of others, in both oysters and mussels I have observed many leucocytes in the lumen of the gut with ingested diatoms; but I have never seen any in the blood or tissue spaces, nor, so far as I am aware, has any one else. However, Gutheil (1912) figures and describes blood cells with food balls passing from the gut into the connective tissues. Yonge (1926b) did report that cells with ingested fat moved back into the blood spaces and tissues in *O. edulis*. I cannot confirm it for *C. virginica*. Wandering cells with lipoidal inclusions characteristically present in the connective tissues may have misled observers. As pointed out elsewhere, these lipoidal inclusions in *C. virginica* differ from the experimental fat in certain respects: they stain blue-green instead of blue with Nile blue sulphate; they do not stain with alcoholic Sudan III, and they are insoluble in absolute alcohol and xylene.

In spite of the evidence for digestion and absorption of stained emulsified fat, why was no evidence obtained in regard to the deposition of stained fat in the tissues of oysters and mussels? Gage and Fish (1924) got clear-cut results with birds and mammals and were able to follow stained fat through two generations. Three possible explanations come to mind: 1) oysters and mussels may be unable to re-synthesize the fatty acids of olive oil and peanut oil into their own specific fat, since the fatty acids of these oils differ from the unsaturated fatty acids of plankton on which the lamellibranchs feed (Markley, 1947); 2) fat may be deposited in connective tissue cells in such small discrete units that the pink color of the droplets is not distinguishable under the microscope, and the total amount of fat in an area may not be massive enough to be detectable on gross examination; 3) at the time of these experiments the stained fat absorbed may have been rapidly metabolized rather than stored. At certain periods, during the summer or after the development of spawn or fattening in the fall, fat is apparently rapidly metabolized. Nelson (1951) reports that great quantities of fat and oil can be demonstrated in New Jersey oysters in late spring before the development of spawn, and in late summer and early fall prior to the "fattening" which follows summer spawning. He suggests that this may be preliminary to the rapid build-up of glycogen, in which the storage in the gland of substantial quantities of oil constitutes the first step. This point deserves further investigation.

Summary

Emulsions of olive oil and peanut oil stained with Sudan stains were fed to oysters and mussels and mixed with mince of crystalline styles and other structures. Samples of the experimental material were subsequently examined to determine if the fat was absorbed and how, and counterstained with Nile blue sulphate to determine if the neutral fat had been split to fatty acid and glycerine. The following observations and conclusions resulted:

1. Free droplets of neutral fat are hydrolyzed in the stomach.

2. Droplets of the stained fat in the form of fatty acid appeared in large numbers in the ciliated epithelium of the stomach and ducts of the digestive gland. None was found in the non-ciliated epithelium of the alveoli of the digestive gland.

3. Droplets of the emulsion were ingested by leucocytes in the lumen of the gut. Neutral fat in leucocytes was hydrolyzed. There was no certain evidence of the passage of any leucocytes with ingested fat back into the blood spaces or tissues.

4. Emulsion injected into the cavity of the heart or mixed in hanging drops of blood was ingested by leucocytes. Hydrolysis occurred within blood cells. No evidence was found for the hydrolysis of neutral fat by the plasma.

5. Tests of stained emulsion subjected to the influence of minced styles showed hydrolysis of neutral fat in all cases.

6. Emulsion subjected to the influence of minced digestive gland was positive for the hydrolysis of neutral fat in some species, but not in others.

7. No evidence for deposition of stained fat in the tissues was found. Possible explanations are suggested.

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